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Intraspecific Variation in Four Distinct Populations of *Anemia villosa* Humb. & Bonpl. ex Willd. (Anemiaceae) Occurring in Rio de Janeiro, Brazil

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Abstract.—Leaf samples of *Anemia villosa* (Anemiaceae) were collected in four distinct places in Rio de Janeiro State, Brazil. Two coastal land populations are located in Itacoatiara and Imbuhy rocky outcrops, municipality of Niterói, and two populations in the inland mountain region of the state, Pedra Dubois, municipality of Santa Maria Madalena, and in Lumiar, municipality of Nova Friburgo. Five leaves of each one of five randomly chosen individuals were collected in each site. The four populations analyzed were very similar in anatomic qualitative characteristics. ANOVA results for the quantitative variables showed significant differences between all populations. The PCA separated the populations analyzed in two groups: one formed by the coastal land and other formed by the inland mountain.

Key Words.—Intraspecific variation, *Anemia*, fern, leaf anatomy

The diversity of plant communities that comprise the Brazilian Atlantic rain forest complex has prompted a number of studies on functional variation of species that are widespread in various habitats within this complex. All of these studies have indicated a large degree of intraspecific variation between distinct populations at different habitats, regarding both anatomical and physiological traits (Scarano *et al.*, 2002; Scarano *et al.*, 2005; Mantuano *et al.*, 2006).

Anatomical and morphological characters may serve as reliable indicators in the study and understanding of ecological adaptations of living organisms (Fahn, 1964). Environmental conditions, such as light intensity and nutrient availability, can influence the size and shape of leaves as well as anatomical aspects. For example, in high light habitats, leaves of many plants tend to be smaller and more deeply incised than under shadier conditions (MacLellan, 2000). In addition, there are other well known shifts in leaf traits such as

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cuticle thickness, epidermal cell size, stomatal frequency and length, and trichome frequency and length that are often related to environmental conditions (Olson and Carlquist, 2000; Rôças *et al.*, 2001; Hlwatika and Bhat, 2002).

Studies on fern morphology and anatomy are common (Kraus *et al.*, 1993, Graçano *et al.*, 2001; Chaerle and Viane, 2004; Pita *et al.*, 2006a, b), however studies on intraspecific variation in such traits in ferns are not usual (Liu *et al.*, 2006; Boeger *et al.* 2007). According to Mickel (1962), in the last hundred years, *Anemia* has been an explored subject for morphological studies that have been largely concerned to the taxonomy. Little work, other than that of Ribeiro *et al.* (2007), has been done on understanding how environmental conditions influence morphology in the genus *Anemia*.

Anemia villosa Humb. & Bonpl. ex Willd. is widely distributed in eastern Brazil, from Santa Catarina to Ceará, and also in northern South America from Peru to Surinam. In Rio de Janeiro State, large populations are commonly associated with vegetation islands on rocky outcrops (Santos and Sylvestre, 2006). According to Mickel (1962) this species has a considerable variation in size and form that can lead to confusion with other species. In Rio de Janeiro State, *A. villosa* populations exhibit two different specimen sizes. The aim of this study was to analyze anatomical variation and environmental parameters in *A. villosa* growing at four different sites in Rio de Janeiro State, Brazil to generate a better understanding of the degree of plasticity exhibited by this species across a variety of habitats.

Material and Methods

Leaf samples of *Anemia villosa* were collected in four distinct sites in Rio de Janeiro State, Southeastern Brazil: Pedra de Itacoatiara (Serra da Tiririca State Park) and Forte Imbuhy, on the coast of Niterói; Pedra Dubois at municipality of Santa Maria Madalena and Lumiar, municipality of Nova Friburgo. All populations grew in inselbergs, except for Lumiar, which was collected at the border of the Atlantic rain forest (Table 1). The two populations in municipality of Niterói are approximately 14 km apart from each other.

In each collection site, five plants were randomly chosen, except for the site of Pedra Dubois, where only three plants were selected. From each plant, five leaves were collected and immediately fixed in formalin-acetic acid-alcohol 70% (FAA_{70}) (Berlyn and Miksche, 1976). Fragments of each last pinna (0.5 cm^2) were gradually dehydrated in ethanol and embedded in glycol methacrylate resin (Feder and O'Brien, 1968). Five micrometer thick transverse sections were made with a rotary microtome, and were subsequently stained with 0.1% toluidine blue O (O'Brien and McCully, 1981). The tracheary elements were observed in material dissociated using the Franklin method (1945) and stained by hydroalcoholic safranin (Johansen, 1940). Identification of vascular bundle types followed Ogura (1972) and stomata Sen and De (1992).

TABLE 1. Climatic aspects of the four sites from where the samples of *Anemia villosa* Humb. & Bonpl. ex. Willd were collected (Rio de Janeiro-Brazil).

	Pedra Dubois	Lumiar	Forte Imbuhy	Pedra de Itacoatiara
Municipality	Santa Maria Madalena	Nova Friburgo	Niterói	Niterói
Sites	Inselberg	Atlantic Rain Forest (bordering)	Inselberg	Inselberg
Latitude e Longitude	21°45′S 41°41′W	22°21′S 42°27′W	22°56′S 43°07′W	22°58′S 43°01′W
Altitude	1090 m	1700m	50m	200m
Mean Temperature	20°C	18°C	23°C	23°C
Mean annual rainfall	1440mm	2205mm	1207mm	1207mm

Anatomical observations and measurements were made using an Olympus BX50 light microscope with the aid of Image-Pro Plus version 4 software. The images were acquired by a video camera Cool SNAP-Pro.

The following leaf anatomical parameters were measured for each sample: thickness of leaf blade, mesophyll, adaxial and abaxial epidermis; trichome length and density; stomata length and density; and lignified petiole area. Means and standard deviations were calculated from a sample size of 25 fields for each measurement (N=25). A nested ANOVA was used to study the association between the outcome variable and group. Principal Component Analysis (PCA) was used to find the higher variance components. To test the hypothesis that the analyzed sample was composed of morphologically different discrete groups, a Discriminant Function Analysis (DFA) was performed (Schlichting, 1986; Zar, 1996). Overall differences between the compared groups are presented by Mahalanobis distance. The statistical analyses were performed with Statistica v. 6.0 (Zar, 1996).

RESULTS

As described by Mikel (1962), different populations of *Anemia villosa* show differences in leaf morphology. The specimens from Lumiar and Pedra Dubois exhibit the typical oblong leaf blade with fertile pinnae close to the sterile pinnae, and the specimens from Niterói have triangular leaf blade with fertile pinnae that vary in their localization from remote to close to the sterile pinnae (Fig. 1).

The four populations analyzed are similar in qualitative characters such as type of stomata, trichomes, and stele. The petiole has a uniseriated epidermis with lignified walls along with uniseriated trichomes. The stomata are parallel in the adaxial epidermis, making up two lines, and are supported above the common epidermal cells by cells with lignified walls. Below the epidermis there are four to five layers of sclerenchyma followed by parenchyma with some amyloplasts. The stele is V-shaped and the endodermis is clearly visible and shows evident Casparian strips; two layers of pericycle were observed.

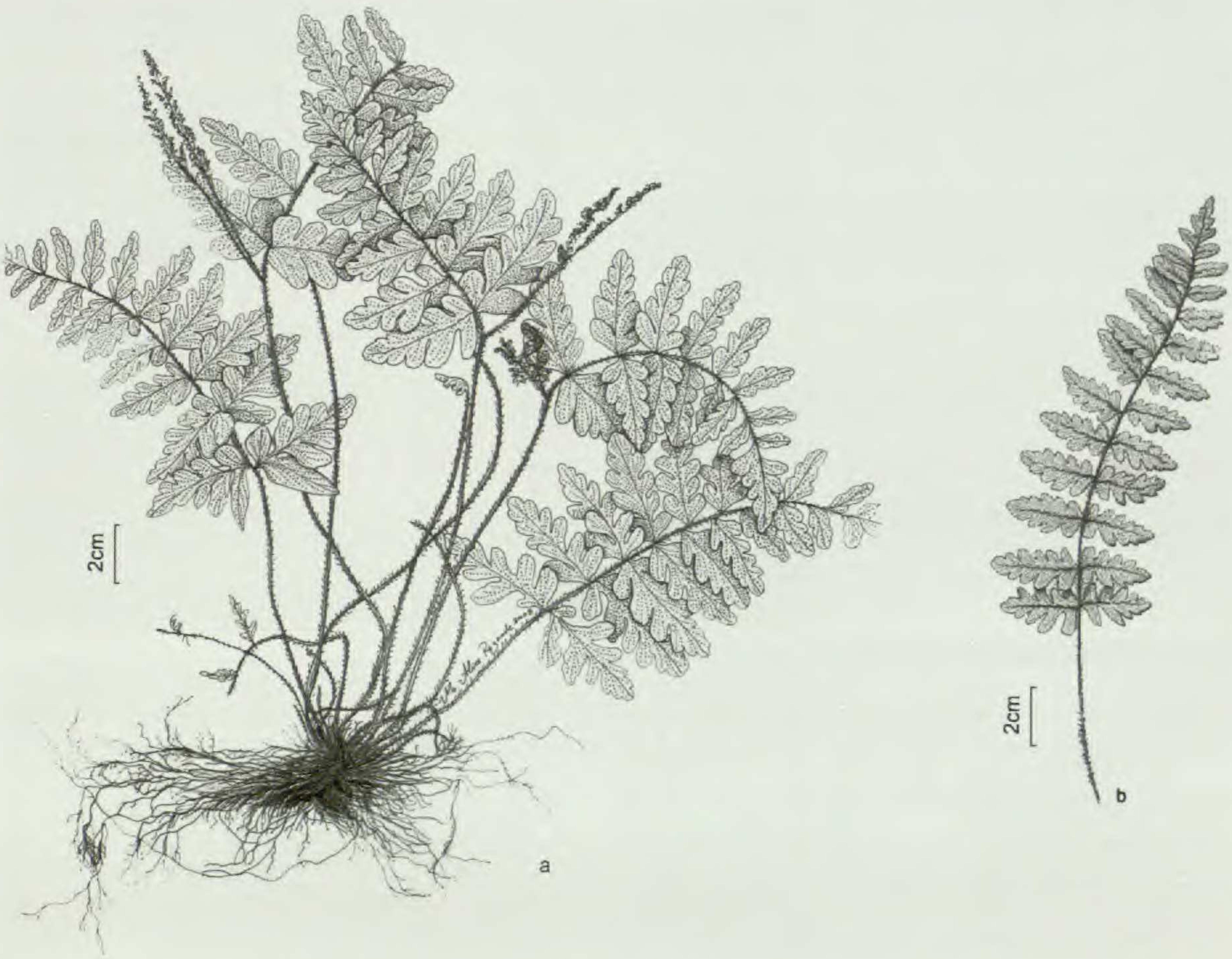


FIG. 1. Specimens of *Anemia villosa* Humb. & Bonpl. ex Willd. from Pedra de Itacoatiara (a) and Pedra Dubois (b).

The xylem showed incurved margins and consists of scalariform tracheids and parenchyma cells. The sieve elements were observed almost completely surrounding the xylem; the phloem consists of sieve cells and parenchyma (Fig. 2).

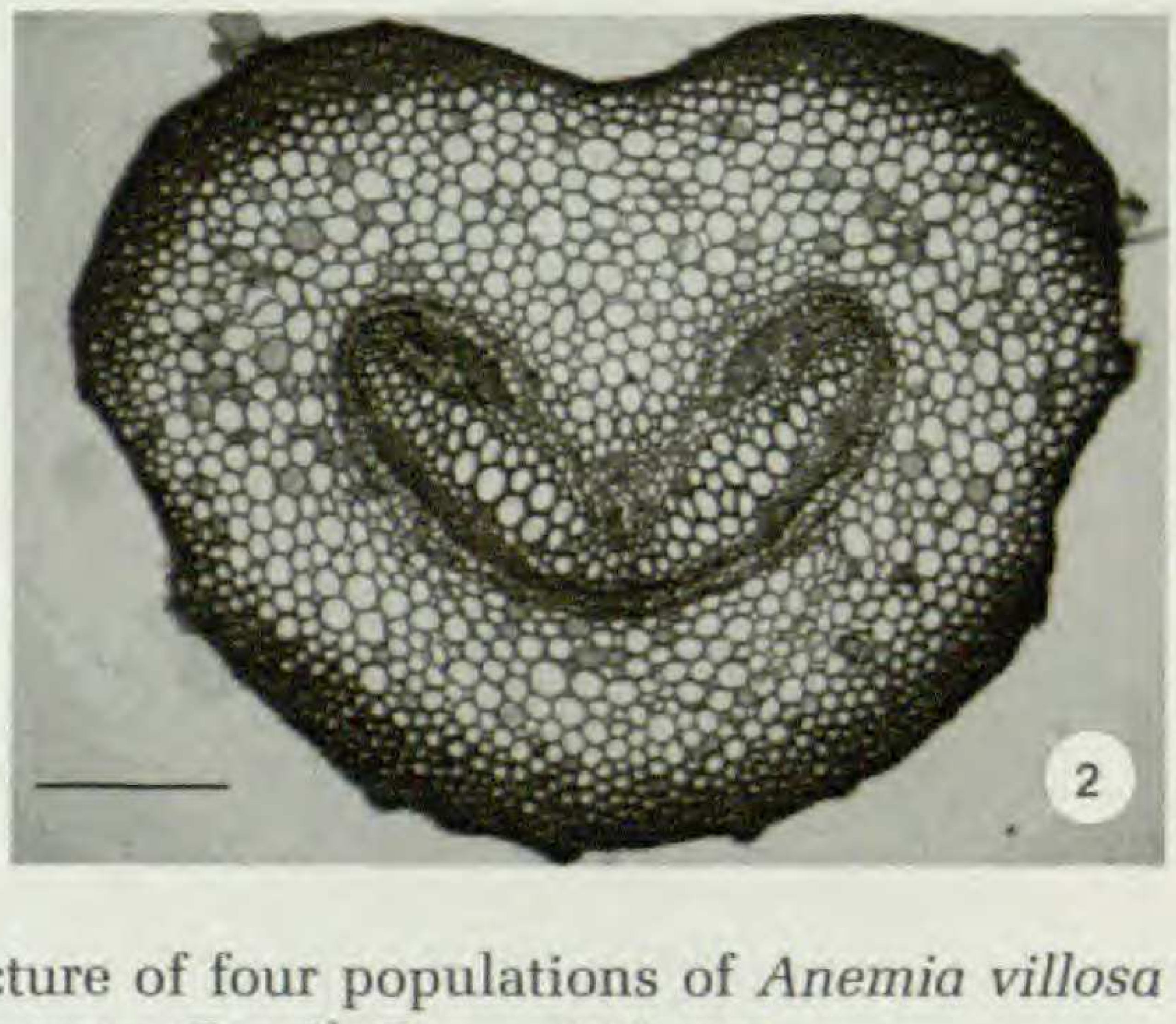
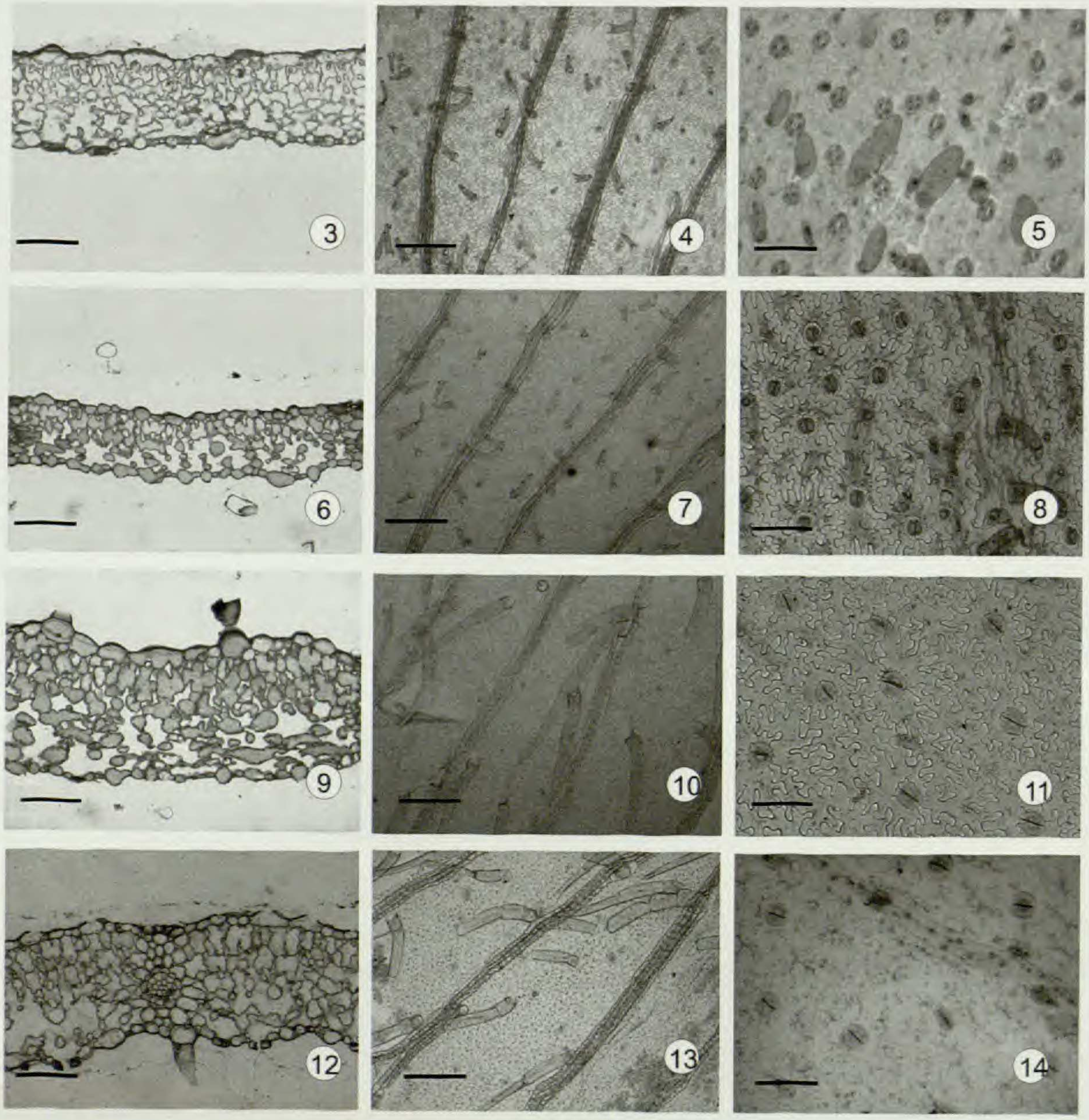


FIG. 2. Basic petiole structure of four populations of *Anemia villosa* Humb. & Bonpl. ex Willd. occurring in Rio de Janeiro state, Brazil. Bar = 350 mm.



FIGS. 3–14. Sections of leaves of four populations of *Anemia villosa* Humb. & Bonpl. ex Willd. occurring in Rio de Janeiro state, Brazil. 3–5. Leaf of Itacoatira populations. 3) Cross section of leaf blades. 4) Adaxial epidermis surface. 5) Abaxial epidermis surface. 6–8. Leaf of Imbuhy populations. 6) Cross sections of leaf blades. 7) Adaxial epidermis surface. 8) Abaxial epidermis surface. 9–11. Leaf of Lumiar populations. 9) Cross sections of leaf blades. 10) Adaxial epidermis surface. 11) Abaxial epidermis surface. 12–14. Leaf of Pedra Dubois populations. 12) Cross sections of leaf blades. 13) Adaxial epidermis surface. 14) Abaxial epidermis surface. Bars = 10 μm in Figs. 3, 5, 6, 8, 9, 11, 12, 14; 200 μm in Figs. 4, 7, 10, 13.

All populations have hypostomatic leaves. The epidermis is comprised of one layer of cells with sinuous anticlinal walls and convex periclinal walls, in both abaxial and adaxial surfaces. The stomata are desmocytic and polocytic, and the guard cells of the stomata protrude a little from the surface of the epidermis. Pluricellular trichomes are present in both adaxial and abaxial surfaces. The mesophyll shows 3–4 layers of arm-cells, which are more compactly arranged near the adaxial epidermis (Figs. 3–14).

TABLE 2. Leaf anatomical differences of ecological populations of *Anemia villosa* Humb. & Bonpl. ex Willd (Mean ± standard deviation) and F values (NESTED-GLM).

	Pedra Dubois	Lumiar	Forte Imbuí	Pedra de Itacoatiara	F
Epidermis thickness of adaxial surface (µm)	21.28±6.3a	23.59±4.55a	21.34±4.83a	21.11±4.8a	8.19
Epidermis thickness of abaxial surface (µm)	23.95±5.85a	21.15±5.22ab	21.32±5.03ab	20.23±5.26b	14.07
Trichomes length of adaxial surface (µm)	512.12±84.98a	464.25±101.28a	191.50±53.52b	127.19±44.34b	446.77
Trichomes length of abaxial surface (µm)	507.28±92.80a	513.10±99.56a	149.09±40.73b	128.28±41.33b	25.81
Trichomes density of adaxial surface (mm)	6.57±1.73a	10.28±0.97b	18.63±2.4c	17.42±2.4d	520.51
Trichomes density of abaxial surface (mm)	13.57±3.63a	17.08±2.15a	56.96±5.18b	57.64±5.82c	121.95
Length of stomata (µm)	53.08±4.52a	52.99±6.01a	39.15±4.32b	41.48±3.95b	50.06
Density of stomata (mm)	47.83±3.56a	49.08±2.66a	72.65±7.22b	50.48±8.2c	296.10
Leaf thickness (µm)	207.88±16.61a	239.49±39.2b	159.38±21.71c	181.90±15.68d	187.84
Mesophyll thickness (µm)	155.53±11.61a	184.75±40.76b	113.15±17.65c	136.89±12.96d	170.72

The results of the nested ANOVA were significant among sites (F =115.67) and significant among individuals (F=5.66). Nested ANOVA results for the quantitative variables among groups are presented in Table 2. All populations were similar for one out of 10 variables (epidermis thickness of adaxial surface). All populations were different in three variables (trichome density on adaxial surface, leaf thickness and mesophyll thickness). The inland mountain (Pedra Dubois and Lumiar) and coastal land (Pedra de Itacoatiara and Forte Imbuhy) populations were statistically different in three variables (trichomes lengh of adaxial surface, trichomes length of abaxial surface and stomata length).

Factor loadings and eigenvalues for the first two components (PCs1 and 2) extracted in the PCA are shown in Table 3. These accounted for 57.71% of the total variance. Anatomical characters such as trichome density of abaxial surface, uniseriate trichome length of adaxial surface, uniseriate trichome length of abaxial surface, and trichome density of adaxial surface showed the highest (either positive or negative) correlations with PC1, and epidermis thickness of adaxial and abaxial surface showed the highest correlations with PC2. Figure 15 shows that PCA separated the populations analyzed in two groups: one formed by the coastal land populations of Imbuhy and Itacoatiara, and other formed by the inland mountain populations of Parque Estadual do Pedra Dubois and Lumiar.

Factor structure for the 10 variables and eigenvalues for the first two factors (DF1 and 2) extracted in the DFA are shown in Table 3. DF1 was positively

TABLE 3. Factor loadings in PCA and Factor structure coefficients in DFA for the variables of *Anemia villosa* Humb. & Bonpl. ex Willd.

Variables	PC1	PC2	DF1	DF2
Stomata length	−0.761	0.131	0.162	−0.070
Stomata density	0.597	−0.349	−0.184	0.855
Leaf thickness	−0.811	−0.301	0.191	0.053
Trichome density on adaxial surface	0.846	−0.197	−0.341	−0.074
Trichome density on abaxial surface	0.948	−0.827	−0.593	−0.293
Epidermis thickness on adaxial surface	−0.163	−0.696	0.014	0.0115
Epidermis thickness on abaxial surface	−0.169	0.548	0.021	−0.097
Unisseriate trichome length on adaxial surface	−0.881	0.019	0.272	0.367
Unisseriate trichome length on abaxial surface	−0.945	−0.067	0.354	0.278
Lignified area in petiole	−0.852	−0.094	0.190	0.094
Eigenvalues	6.34	1.12	2.84	1.53

correlated with trichome lengh of abaxial surface and negatively correlated with trichome density of abaxial surface, whereas DF2 was positively correlated with stomata density. DFA classification was 98.27% for individuals from groups I, II and III (Fig. 16).

DISCUSSION

In this study all populations of *A. villosa* showed similar qualitative characters but variation was demonstrated in the quantitative characters. This quantitative variation occurred between specimens at different latitudes, temperatures and altitudes in the Rio de Janeiro State showing two distinct groups: the coastal land populations and the inland mountain populations. These two groups were described by Mickel (1962) in Rio de Janeiro state as specimens with oblong leaf blades and fertile pinnae approximate to the sterile pinnae (inland mountain population) and those with broader leaf blades with fertile pinnae varying in their origin from remote to approximate to the sterile pinnae (coastal land populations).

Other studies have verified quantitative variation in populations of angiosperm species that occur in different habitats or micro-habitats (e.g., Rôças *et al.*, 1997, 2001; Hlwatika and Bhat, 2002; Mantuano *et al.*, 2006). The anatomical differences in *Anemia villosa* are evident in traits such as trichome density of adaxial and abaxial surfaces, leaf thickness and mesophyll thickness between populations and in trichome length of adaxial and abaxial surfaces and stomata length between the inland mountain and coastal land populations.

The inland mountain populations, submitted to lower temperatures and higher humidity, presented thicker leaves with longer stomata and trichomes, and low stomata frequency when compared to coastal land populations. The difference in thickness of the leaf and mesophyll was expressed in length but

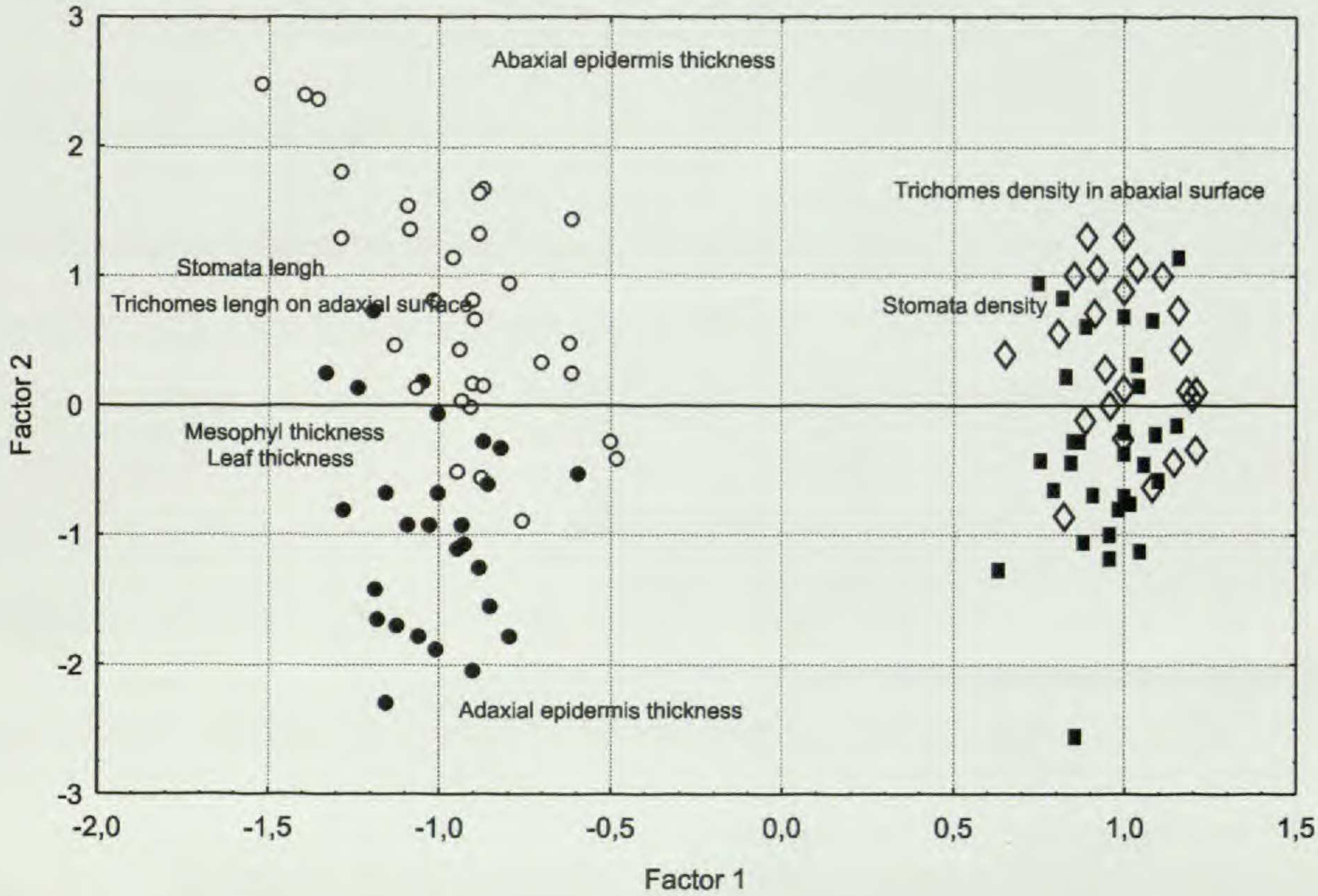


FIG. 15. Principal Components Analysis of four populations of *Anemia villosa* Humb. & Bonpl. ex Willd. occurring in Rio de Janeiro state, Brazil.

not in number of cell layers. According to some studies (Woodward, 1979; Willians and Black, 1993; Körner, 1999; Loeys *et al.*, 2002) biomass allocation is temperature sensitive, and exposure to low temperatures results in plants that exhibit reduced investment in the shoot and leaves that are thicker than their warm-growth counterparts. Differences in stomata frequency were also observed by Hlwatika and Bhat (2002) studying *Rapanea melanophloes* (L.) Mez and *Cunonia capensis* L. in distinct ecological sites. These authors suggest that the higher stomatal frequencies in sclerophyllous vegetation may be a reaction to the favorable photosynthetic conditions.

Rôças *et al.* (1997, 2001), studying intraspecific variation in *Alchornea triplinervia* (Spreng.) Müll. Arg., Mantuano *et al.* (2006) verifying intraspecific variation in *Erythroxylum ovalifolium* Peyr., and Pereira *et al.* (2009) studying variation of *Andira legalis* (Vell.) Toledo leaves, observed differences in epidermis thickness between the populations and related this to changes in light regimes. In *A. villosa* all populations analyzed are fully exposed to sunlight, which may explain why there is no difference in their epidermis thickness.

Although intraspecific variation in ferns has been poorly explored, it is crucial to understand the distribution of this group. According to Liu *et al.* (2006), the leaf anatomy of *Isoetes* shows less environmentally induced variation than the external leaf morphology. In this present study, however, it

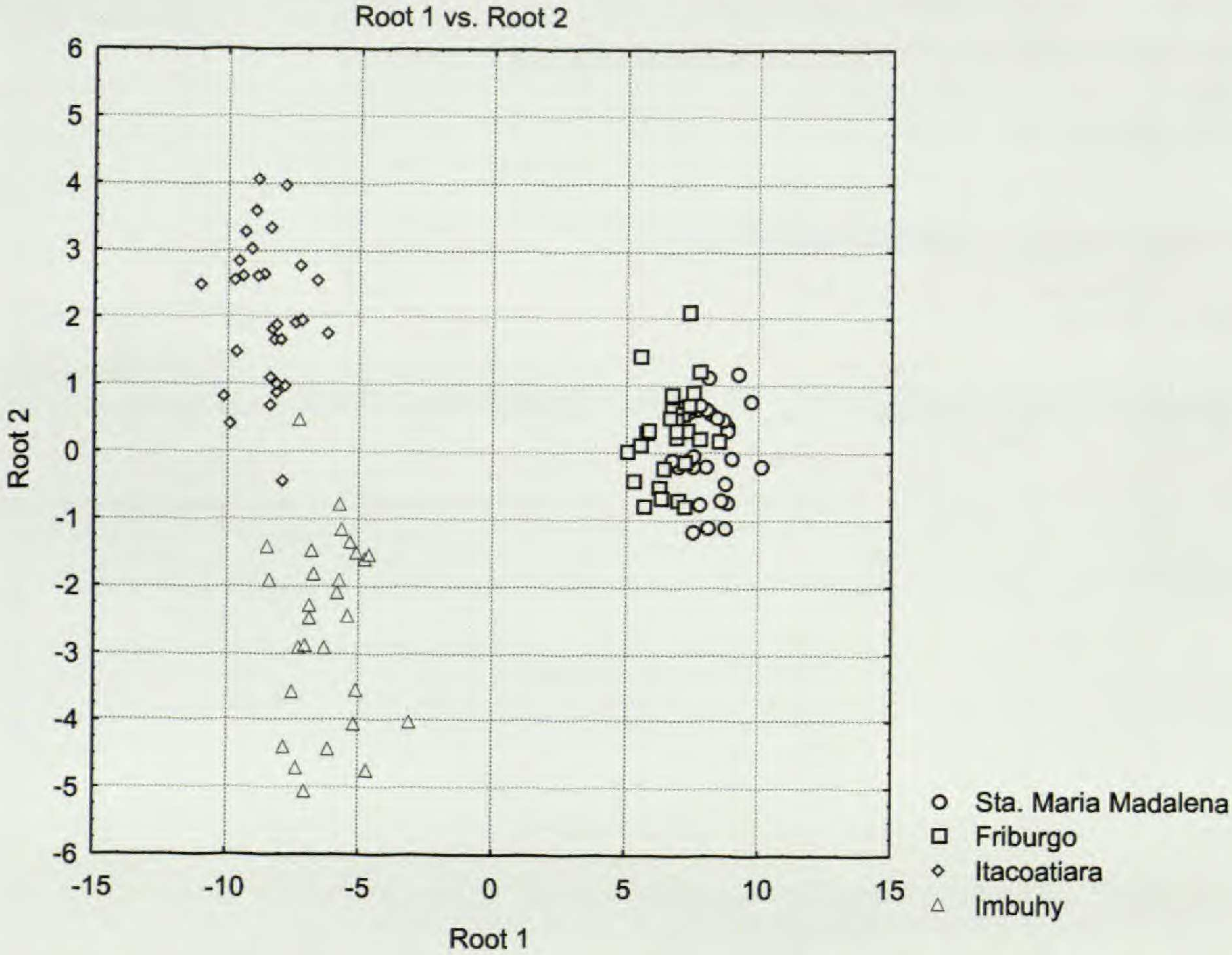


FIG. 16. Discriminant Function Analysis in four populations of *Anemia villosa* Humb. & Bonpl. ex Willd. occurring in Rio de Janeiro state, Brazil.

was verified that under different environmental conditions *Anemia villosa* populations showed both anatomical and morphological modifications.

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